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Distinct tRNA modifications in the thermo-acidophilic archaeon, *Thermoplasma acidophilum*

Chie Tomikawa [a], Takayuki Ohira [b], Yasushi Inoue [c], Takuya Kawamura [a], Akihiko Yamagishi [c], Tsutomu Suzuki [b], Hiroyuki Hori [a,d,*]

[a] Department of Materials Science and Biotechnology, Graduate School of Science and Engineering, Ehime University, Bunkyo 3, Matsuyama, Ehime 790-8577, Japan
[b] Department of Chemistry and Biotechnology, Graduate School of Engineering, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-8656, Japan
[c] Department of Molecular Biology, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
[d] Venture Business Laboratory, Ehime University, Bunkyo 3, Matsuyama, Ehime 790-8577, Japan

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ABSTRACT

***Thermoplasma acidophilum* is a thermo-acidophilic archaeon. We purified tRNA^{Leu} (UAG) from *T. acidophilum* using a solid-phase DNA probe method and determined the RNA sequence after determining via nucleoside analysis and m^{7}G-specific aniline cleavage because it has been reported that *T. acidophilum* tRNA contains m^{7}G, which is generally not found in archaeal tRNAs. RNA sequencing and liquid chromatography–mass spectrometry revealed that the m^{7}G modification exists at a novel position 49. Furthermore, we found several distinct modifications, which have not previously been found in archaeal tRNA, such as 4-thiouridine9, archaeosine13 and 5-carbamoylmethyuridine34. The related tRNA modification enzymes and their genes are discussed.**
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1. Introduction

Thermoplasma acidophilum is a thermo-acidophilic archaeon, which optimally grows at 59 °C and pH 1.9 [1,2]. There is little known with regard to the RNA modifications of this archaeon except for early studies [3–5]. In 1981 and 1983, sequences of initiator and elongator tRNA^{Met} were determined and novel modifications at positions 15 and 56, which were later named archaeosine15 [6] and Cm56 [7–9], were reported [3,4]. In 1991, Edmonds et al. reported that a tRNA mixture from *T. acidophilum* contains N^7-methylguanine (m^{7}G) [5], which is commonly found at position 46 in class I tRNAs (regular tRNAs) from eubacteria and eukaryotes [8,9]. The m^{7}G46 modification contributes to maintaining the L-shaped tRNA structure via formation of a m^{7}G46–C13–G22 tertiary base pair. The m^{7}G modification has been found in anticodon-loops of organelle class II tRNAs (tRNAs with a long variable region): m^{7}G34 in mitochondrial tRNA^{Ser} and m^{7}G36 in chloroplast tRNA^{Leu} [8,9].

In this study, we report that the m^{7}G modification exists at the novel position 49 in class II tRNA^{Leu}. Furthermore, we found several other distinct modifications in this tRNA.

2. Materials and methods

2.1. Strain, media, and culture

T. acidophilum HO-62 strain was previously isolated from Hakone, Japan [2]. The culture was performed at 56 °C under microaerophilic conditions as described previously [2].

2.2. Preparation of class I and class II tRNA fractions, nucleoside analysis and aniline cleavage at the m^{7}G base

Total RNA was prepared from *T. acidophilum* cells by phenol extraction. Class I and class II tRNA fractions were separated by 10% polyacrylamide gel electrophoresis (PAGE) (7 M urea), visualized with 0.2% toluidine blue staining, and the bands were excised. RNAs were extracted with 400 µl of gel elution buffer (0.5 M ammonium acetate, 10 mM $MgCl_2$, 1 mM EDTA, and 0.1% SDS) for 12 h at room temperature and then recovered by ethanol precipitation. Nucleoside analysis was performed according to our recent

* Corresponding author at: Department of Materials Science and Biotechnology, Graduate School of Science and Engineering, Ehime University, Bunkyo 3, Matsuyama, Ehime 790-8577, Japan. Fax: +81 89 927 9941.
E-mail address: hori@eng.ehime-u.ac.jp (H. Hori).

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reports [10,11]. 0.1 A_{260} units of tRNA was ^{32}P-labeled at the 5′-end with [γ-^{32}P]-ATP and T_4 polynucleotide kinase after bacterial alkaline phosphatase treatment. Reduction by $NaBH_4$ and aniline cleavage were performed as described previously [12–14].

2.3. Purification of tRNA^{Leu} by solid-phase DNA probe and RNA sequencing

A 3′-biotinylated DNA oligomer (5′-GTAAATCCATCGCCTTTGGCCAGTCT biotin-3′) was used. Purification of tRNA^{Leu} by solid-phase DNA probe was performed as described [10,11,14–16]. Eluted tRNA^{Leu} was further purified by 10% PAGE (7 M urea). RNA sequence of tRNA^{Leu} was partially determined using Kuchino's post labeling method with slight modifications [10,17,18]. The ^{32}P-labeled nucleotides were analyzed by two-dimensional thin layer chromatography (2D-TLC) [19].

2.4. Mass spectrometry

20 ng of purified tRNA^{Leu} was digested by RNase T_1 and RNase A, and then analyzed by capillary liquid chromatography (LC)/nano-electrospray ionization mass spectrometry (MS) as described [20]. For detecting pseudouridine (Ψ), derivatization of Ψ by cyanoethylation was performed according to the literature [21]. Nucleoside analysis was performed using 800 ng of *T. acidphilum* tRNA^{Leu} and 40 μg of yeast total RNA prepared from *Δtrm9* strain according to our previous report [22].

3. Results

3.1. Class II tRNA fraction contains m^{7}G nucleoside

In 1991, it was reported that a tRNA mixture from *T. acidophilum* contained N^7-methylguanine (m^{7}G) [5], which is commonly found at position 46 in class I tRNAs from eubacteria and eukaryotes [8,9]. Therefore, we initially assumed that the m^{7}G modification in *T. acidophilum* tRNA was contained in class I tRNA. We prepared class I and II tRNA fractions (Fig. 1A) and performed nucleoside analyses (Fig. 1B). Unexpectedly, we could not detect a m^{7}G peak in the class I tRNA fraction (Fig. 1B upper) whereas a small peak was found at the elution position of m^{7}G in the class II tRNA fraction (Fig. 1B lower). To confirm that m^{7}G is present in the class II tRNA fraction, we performed m^{7}G-specific aniline cleavage (Fig. 1C). This showed that bands derived from m^{7}G-modified tRNA(s) were detected in the class II tRNA fraction (Fig. 1C). In contrast, aniline cleavage did not occur in the class I tRNA fraction, consistent with the results from nucleoside analysis.

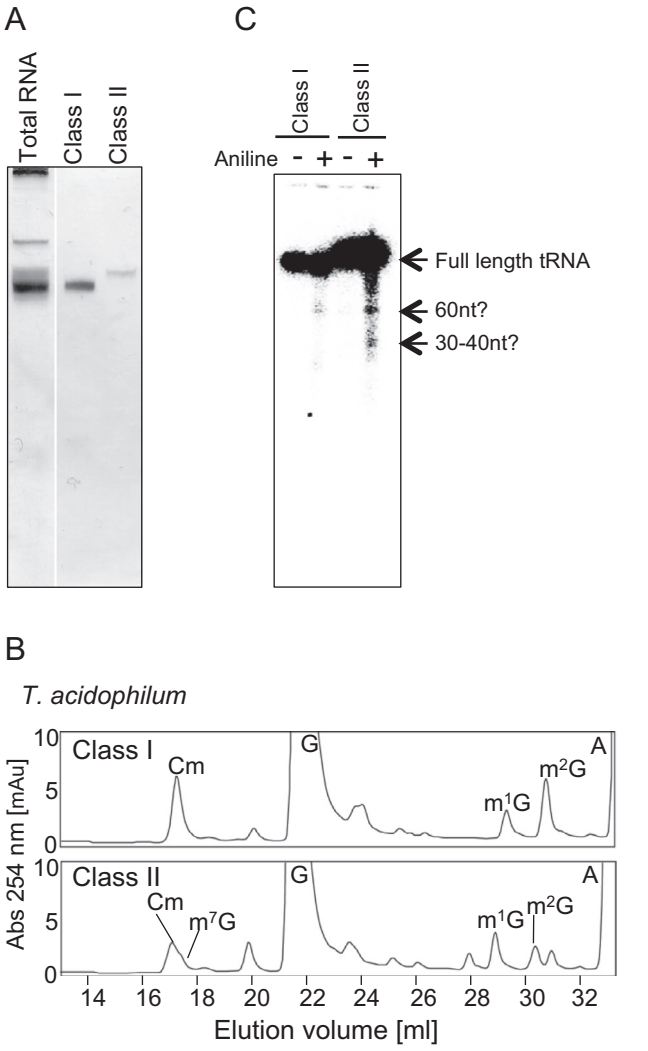


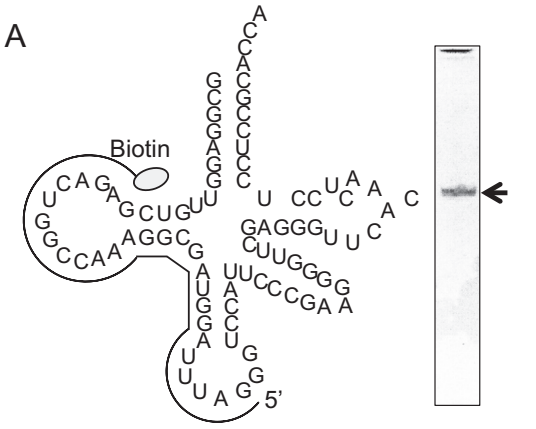
Fig. 1. Class II tRNA fraction from *T. acidophilum* contains the m^{7}G nucleoside. (A) Total RNA fraction was prepared by phenol extraction. Classes I and II tRNA fractions were purified by 10% PAGE (7 M urea). (B) Nucleoside analyses of class I (upper) and class II (lower) tRNA fractions. Classes I and II tRNA fractions were completely digested to nucleosides and then analyzed by HPLC C18 reverse phase column chromatography. A small peak corresponding to m^{7}G was detected in the class II tRNA sample. In contrast, m^{7}G was not detectable in the class I tRNA sample. (C) The 5′-ends of tRNAs were labeled with ^{32}P, reduced by $NaBH_4$, and then treated with aniline. The m^{7}G-specific aniline cleavage was observed in the class II tRNA fraction.

3.2. The m^{7}G modification does not exist in the anticodon of class II tRNAs

Some organelle tRNAs (tRNA^{Ser} and tRNA^{Leu}) have the m^{7}G modification in the anticodon-loop [8,9]. Therefore, we purified tRNA^{Ser} (GCU) and tRNA^{Leu} (UAG) from *T. acidophilum* class II tRNA fraction using a solid-phase DNA probe method (Fig. 2A). Because nucleoside analysis failed to detect m^{7}G in tRNA^{Ser} (data not shown) but showed its presence in tRNA^{Leu} (see Fig. 3A), we analyzed tRNA^{Leu} using Kuchino's post label RNA sequencing method [10,17,18] (Fig. 2B). It is generally difficult to determine the whole sequence of class II tRNA by this method, because the long variable region in class II tRNA shows resistance to formamide limited cleavage. Although we performed the long formamide reaction (120 s), we could not determine the position of m^{7}G. Furthermore, this long reaction caused the increase of double-hit fragments, which gave non-specific pA, pG, pC and pU spots on the 2D-TLC (Fig. 2B). However, we could detect several modifications as shown in Fig. 2B. Unexpectedly, G36 was identified as unmodified (data not shown). Furthermore, we found an unknown uridine modification at position 34. These results prompted us to employ LC/MS analysis of tRNA^{Leu}.

3.3. Mass spectrometry analysis reveals that tRNA^{Leu} contains 5-carbamoylmethyuridine (ncm^{5}U)

Fig. 3A shows the result of the LC/MS analysis. We found a modified uridine at 19.09 min of elution. The *m/z* value of this modified uridine was 302, which coincides with that of 5-carbamoylmethyuridine (ncm^{5}U). The ncm^{5}U modification has been found at position 34 only in tRNA from eukaryotes [8,9,23]. Although the biosynthesis pathway of ncm^{5}U modification has not been clarified, this modification is derived from 5-carboxymethyluridine (cm^{5}U) [24,25]. Although cm^{5}U is the precursor of both ncm^{5}U



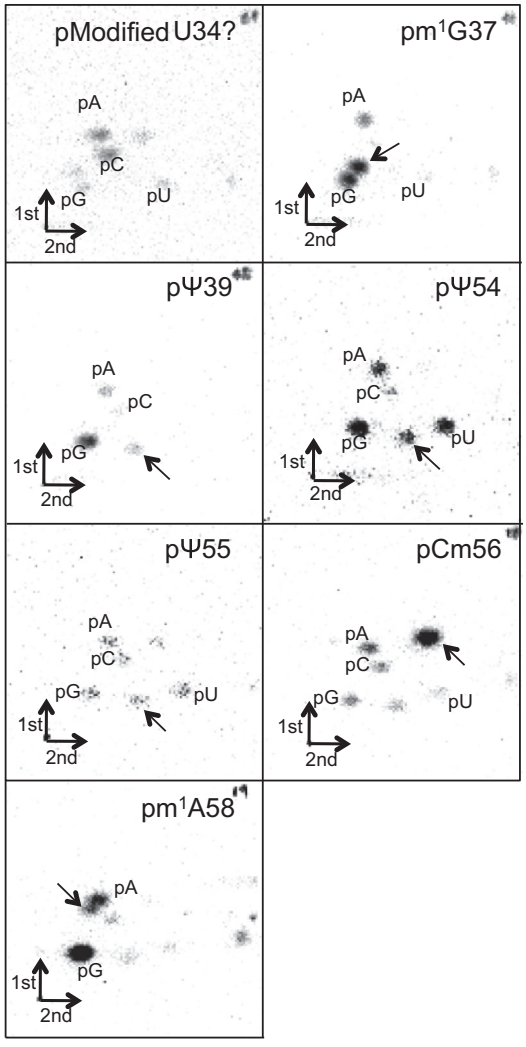
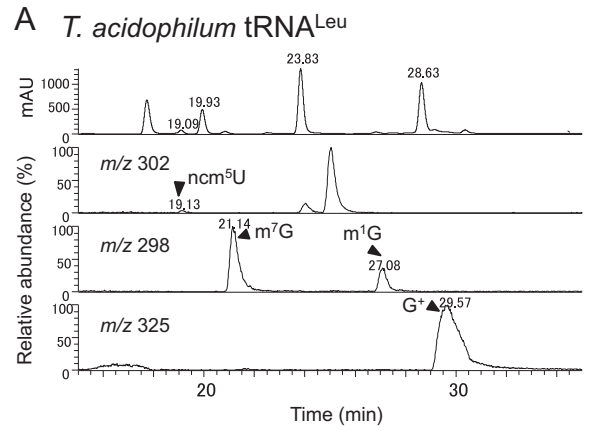
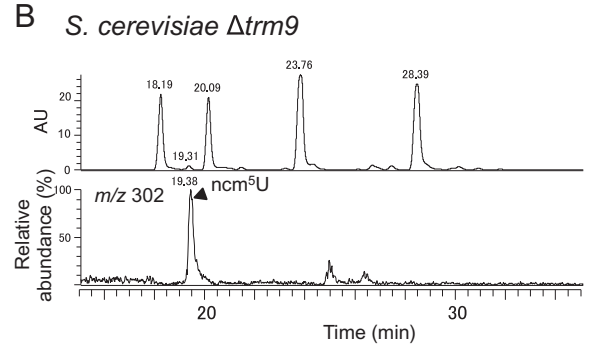


Fig. 2. Purification of $tRNA^{Leu}$ by solid-phase DNA probe and determination of modified nucleotides by Kuchino's post labeling method. (A) Nucleotide sequence of $tRNA^{Leu}$ is depicted as a cloverleaf model. The complementary region to the DNA probe is illustrated. Purity of $tRNA^{Leu}$ was checked by 10% PAGE (7 M urea) (right panel, methylene blue staining). (B) 0.02 A_{260} units of $tRNA^{Leu}$ were analyzed by the Kuchino's post labeling method. Several modified nucleotides could be identified by 2D-TLC. The arrows show the spots corresponding to modified nucleotides. The unidentified spot (depicted as ncm^5U34?) was determined as ncm^5U34 by later analyses (see Figs. 3 and 4).





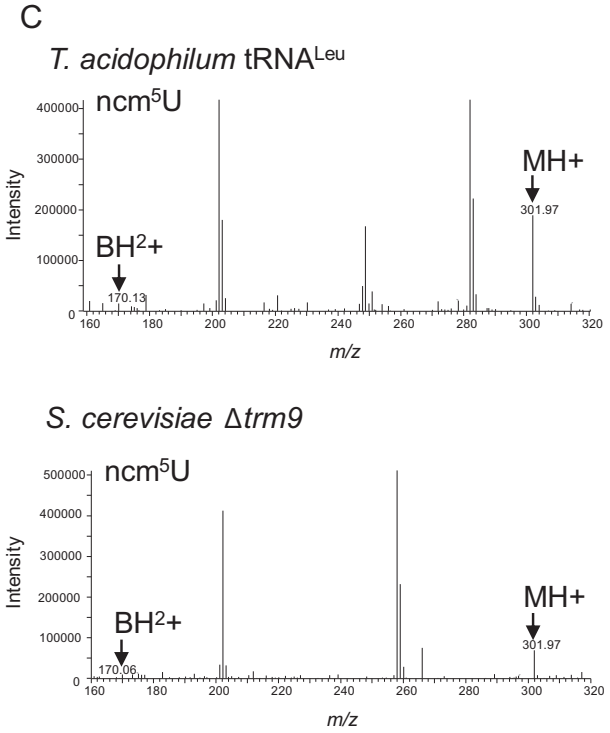


Fig. 3. Nucleoside analysis of $tRNA^{Leu}$ by LC/MS. (A) Purified $tRNA^{Leu}$ was completely digested to nucleosides and then analyzed by LC/MS. The unknown modified U eluted at 19.09 min. The *m/z* value was determined as 302, which coincides with that of ncm^5U. Furthermore, m^7G, m^1G and archaeosine could be detected. (B) To determine the elution position of ncm^5U, we analyzed tRNA from the *S. cerevisiae trm9* gene disruptant strain. The ncm^5U eluted at 19.31 min. (C) The BH^{2+} and MH^+ ions were compared between modified U from *T. acidophilum* $tRNA^{Leu}$ (upper) and the standard ncm^5U marker (lower). The elution times and BH^{2+} and MH^+ ions coincided, demonstrating that the unknown U modification in *T. acidophilum* is ncm^5U.

and 5-methoxycarbonylmethyluridine (mcm^5U), the biosynthesis of mcm^5U requires Trm9 and Trm112 proteins in *Saccharomyces cerevisiae* [25]. Thus, the *S. cerevisiae trm9* gene disruptant strain accumulates ncm^5U in its tRNA fraction [25]. To determine whether the modified U in $tRNA^{Leu}$ is ncm^5U, we prepared the standard ncm^5U from the *S. cerevisiae trm9* gene disruptant strain (Fig. 3B) and compared the modified U and ncm^5U by LC/MS (Fig. 3C). The elution times and BH^{2+} and MH^+ ions coincided between the modified uridine from *T. acidophilum* $tRNA^{Leu}$ and the standard marker of ncm^5U. Thus, we confirmed that *T. acidophilum* $tRNA^{Leu}$ contains ncm^5U. Furthermore, we confirmed the identity of the peak of m^7G in the LC/MS of nucleosides from $tRNA^{Leu}$ (Fig. 3A), consistent with the result of the aniline cleavage experiment (Fig. 1C).

3.4. Distinct modifications in *T. acidophilum* $tRNA^{Leu}$

Positions of modified nucleotides were determined by LC/MS/MS analysis. $tRNA^{Leu}$ was digested with RNase A or RNase T_1 and then the digested fragments analyzed (Supplementary Fig. 1 and Table 1). For example, in the case of the m^7G modification, the modification was found in the fragment UUCG*AGp, which was derived from RNase T_1 digestion (Fig. 4A). Because RNase T_1 cleaves at the 3′-end of G residues, the existence of the UUCG*AGp fragment suggests the modification of G* (corresponding to G49). The mass of the fragment (1968 Da, Supplementary Table 1) suggests the methylation of G*. Two methylguanosines (m^7G and m^1G) were detected by nucleoside analysis (Fig. 3A) and the position of m^1G was determined as 37 by Kuchino's post label method (Fig. 2B), strongly suggesting that the G* is m^7G. In fact, the MS/MS analysis detected the fragment (m/z = 900.594) without the m^7G base derived from UUCG*AGp (Fig. 4A), because the *N*-glycosyl bond of m^7G is unstable as compared to those of the other methylated guanosines [20]. Thus, G*49 was identified as m^7G49. This conclusion was confirmed by LC/MS/MS analysis of RNase A digested sample: the m^7GAGGGUp fragment was detected (Supplementary Fig. 1 and Table 1). Similarly, s^4U8 and s^4U9 (Fig. 4B), archaeosine (G^+) 13 and G^+15 (Fig. 4C), ncm^5U34 (Fig. 4D), $m^2{}_2G26$ (Supplementary Fig. 1 and Table 1), Ψ39 and Ψ55 (Supplementary Fig. 1 and Table 1), Cm56 (Supplementary Fig. 1 and Table 1) and m^1A58 (Supplementary Fig. 1 and Table 1) were detected by LC/MS/MS. The positions of Ψ modifications were determined by LC/MS/MS analysis of cyanoethylated $tRNA^{Leu}$ [21]. Because the reaction efficiency of cyanoethylation at position 54 was low, we could not confirm whether the Ψ54 modification was present. However, the results of Kuchino's post label method clearly showed the existence of Ψ54 modification. Therefore, we concluded that U54 is modified to Ψ54. A summary is shown in Fig. 5 and Table 1.

4. Discussion

In this study, we initially focused on the m^7G modification in *T. acidophilum* tRNA because the position(s) of this modification remained unidentified for two decades [5]. To our surprise, the m^7G modification was found at the novel, irregular position 49 in the class II $tRNA^{Leu}$. *Aquifex aeolicus* TrmB (tRNA (m^7G46) methyltransferase) [14,26] does not methylate this $tRNA^{Leu}$ transcript (data not shown), suggesting that m^7G49 in $tRNA^{Leu}$ is not a result of conformational change of tRNA and an already known tRNA methyltransferase. Thus, this result suggests the existence of a novel tRNA (m^7G49) methyltransferase. Because tRNA (m^7G46) methyltransferases (eukaryotic Trm8–Trm82 [27] and eubacterial TrmB [26,28]) share homology in their catalytic domain, we

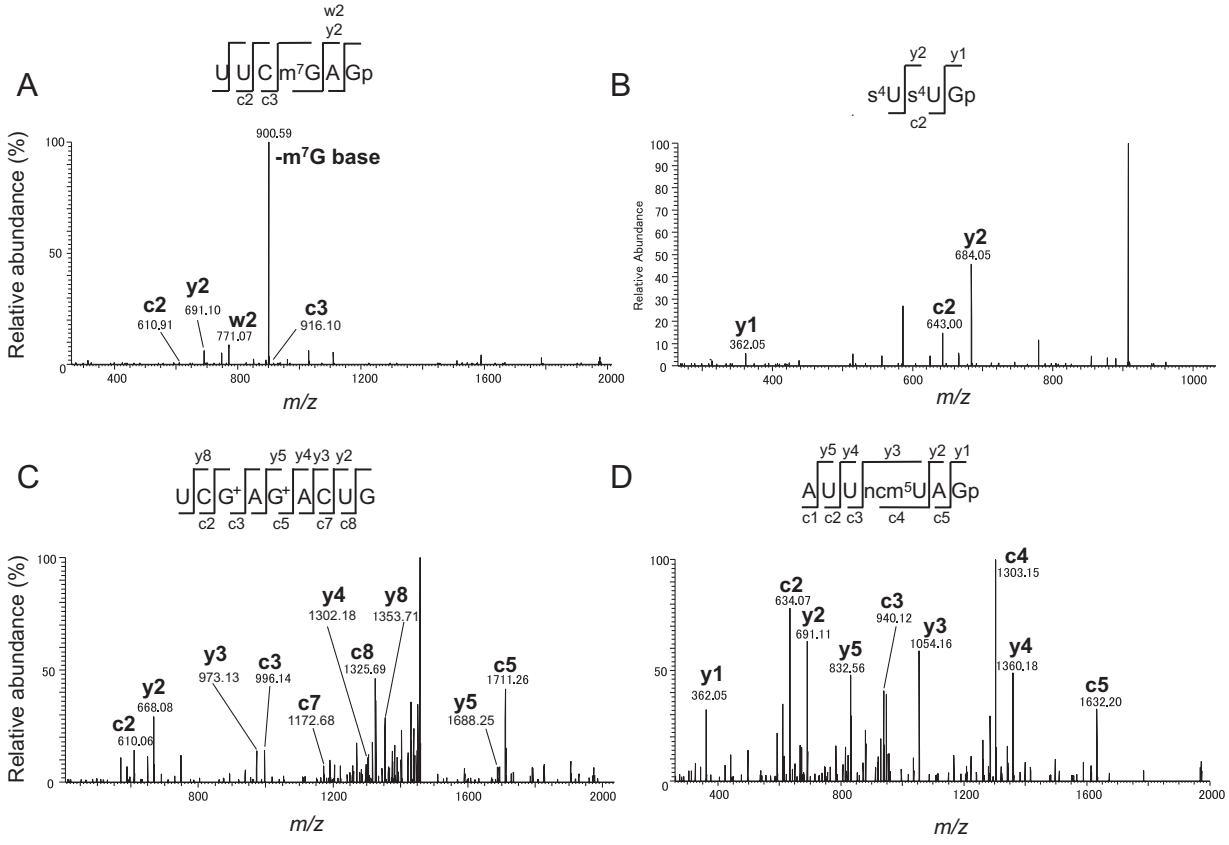
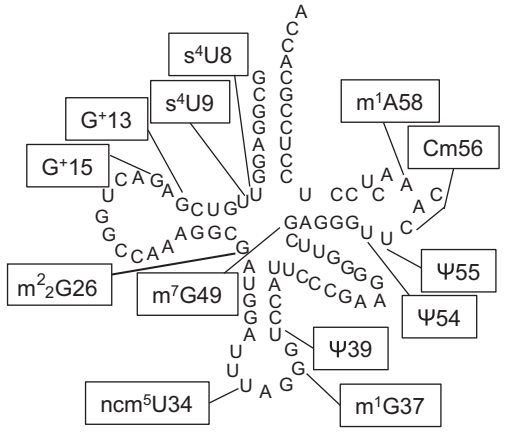


Fig. 4. Determination of positions of modified nucleotides by LC/MS/MS analysis. The purified $tRNA^{Leu}$ was digested with RNase T_1 and analyzed by LC/MS/MS. m^7G49 (A), s^4U8 and s^4U9 (B), G^+13 and G^+15 (C), and ncm^5U34 (D) were identified.



T. acidophilum tRNA^{Leu}(UAG)

Fig. 5. Modified nucleosides in tRNA^{Leu} (UAG). The positions of modified nucleosides (boxed) are illustrated on the clover-leaf structure. The features and expected modification enzymes are summarized in Table 1.

searched for candidate genes in the *T. acidophilum* genome. Although one candidate gene (Ta0679) was found (Table 1), we have been unable to prepare soluble recombinant protein in *Escherichia coli* (data not shown). The tRNA (m^{7}G49) methyltransferase activity may require another subunit as in eukaryotic Trm8–Trm82.

The s^{4}U modification at position 9 is novel in archaeal tRNAs, whereas this modification is often found in eubacterial class II tRNAs [8,9,29]. The eubacterial s^{4}U8 modification is generated by the cooperative activity of ThiI and IscS, transferring a sulfur atom from cysteine to tRNA [30,31]. Because s^{4}U8 is also found at the neighboring position, ThiI is probably involved in the s^{4}U9 modification. However the *iscS* gene is not present in the *T. acidophilum* genome [32]. ThiI from γ-proteobacteria consists of four domains including a rhodanase-like domain (RLD) [30,31]. In contrast, general archaeal ThiI proteins do not have a RLD [33]. However, *T. acidophilum* ThiI is an exception and does contain a RLD like γ-proteobacterial proteins. This unusual RLD may function instead of IscS. Furthermore, because *T. acidophilum* prefers a sulfur-rich environment, *T. acidophilum* may utilize a sulfur atom for tRNA modification without IscS like the ThiI from *Methanococcus maripaludis* [33]. Moreover, it has been reported that ubiquitin-like small proteins are involved in the sulfur transfer in archaea: at least UbaA protein from *Haloferax volcanii* is involved in 2-thiolation of U34 [34]. These ubiquitin-like small proteins may be involved in s^{4}U formations in *T. acidophilum*. Because IscS is involved in thiamine biosynthesis [31,35], future study is required to investigate sulfur metabolism in *T. acidophilum*.

Unexpectedly, G$^+$ was detected not only at position 15 (common position) but also at position 13 (novel position). In general, G$^+$ is synthesized by two-step reactions [36] involving ArcTGT and ArcS. Only one set of these genes is encoded in the *T. acidophilum* genome and are therefore probably involved in the biosynthesis of G$^+$13 as well as G$^+$15.

The ncm^{5}U34 is a novel modification in archaeal tRNAs. Although the biosynthesis pathway of ncm^{5}U34 is unclear, the elongator complex is involved in the eukaryotic ncm^{5}U34 modification [24]. The eukaryotic elongator complex is composed of six subunits whereas only one gene (*elp3*) exists in the *T. acidophilum* genome. Archaeal Elp3 consists of a *S*-adenosyl-L-methionine binding central domain and a C-terminal histone acetyltransferase-like domain [37]. This archaeal Elp3 may be involved in the biogenesis pathway of ncm^{5}U34. In general, xm^{5}U modifications at the first position (i.e., 34) in the anticodon shifts the puckering equilibrium of the ribose of xm^{5}U34 to the C3′-endo form and results in restriction of the wobble base pairing only with A and G [38]. In the *T. acidophilum* genome, five tRNA^{Leu} genes exist and one tRNA^{Leu} has a CAG anticodon. Therefore, there is no necessity to modify U34 to ncm^{5}U34. These observations may mean that there is a division of roles of the five tRNA^{Leu} species according to the corresponding codons in *T. acidophilum* protein synthesis.

The m$^2_2$G26 modification has not been reported from archaeal class II tRNAs [8,9]. In fact, it has been reported that one of positive determinants for *Pyrococcus furiosus* Trm1 is the regular size variable region (5 nt) [39]. In contrast, Trm1 from *A. aeolicus* (thermophilic eubacterium) can methylate the class II tRNA [15]. Therefore, the substrate specificity of *T. acidophilum* Trm1 seems to be a eubacterial type.

The other modifications are explainable by reported archaeal tRNA modification enzymes (Table 1) [40–43]. Furthermore, although we detected the m^{1}A58 modification in tRNA^{Leu}, A58 in elongator tRNA^{Met} has been reported as unmodified A58 [3]. In addition, *Pyrococcus abyssi* TrmI has a multisite specificity towards A57 and A58 [42]. In the current study, we did not detect the m^{1}A57 modification in tRNA^{Leu}. Therefore, *T. acidophilum* TrmI may modify specific tRNA species. To characterize these tRNA modification enzymes, further studies will be necessary.

Genome sequencing demonstrated that *T. acidophilum* genome contains only around 1500 open reading frames [32]. Therefore, we initially imagined the *T. acidophilum* tRNA modification system as a simplified system formed by limited tRNA modification enzymes. However, contrary to expectations, tRNA modifications in

Table 1
Modified nucleosides in *T. acidophilum* tRNA^{Leu}.

This study	In previous reports			Feature	Expected modification enzyme	Candidate gene(s) in *T. acidophilum*
	Archaea	Eubacteria	Eukaryotes			
s^{4}U8	+	+	–	Archaea and eubacteria specific	UbaA? + ThiI	Ta0844? + Ta0506
s^{4}U9	–	+	–	Novel position in archaea	UbaA? + ThiI?	Ta0844? + Ta0506?
G$^+$13	–	–	–	Novel position	ArcTgt? + ArcS?	Ta1493? + Ta0924?
G$^+$15	+	–	–	Archaea specific	ArcTgt + ArcS	Ta1493 + Ta0924
m$^2_2$G26	+	+	+	Novel position in archaeal class II tRNA	Trm1	Ta0997
ncm^{5}U34	–	–	+	Novel modification in archaea	Elp3? + α?	Ta1311? + α?
m^{1}G37	+	+	+	Common	aTrm5	Ta0836
Ψ39	+	+	+	Common	Pus3	Ta0932
m^{7}G49	–	–	–	Novel position	?	Ta0679? + α?
Ψ54	+	–	+	Archaea and eukaryotes specific	Pus10	Ta1296
Ψ55	+	+	+	Common	Pus10 or Cbf5 with H/ACA proteins (Nop10 and Gar1)	Ta1296 or Ta1244 with (Ta1202 and Ta0940)
Cm56	+	–	–	Archaea specific	aTrm56	Ta0931
m^{1}A58	+	+	+	Common	aTrmI	Ta0852

T. acidophilum are unprecedented. Because genome sequencing suggests that more than 200 genes are derived from other archaea and eubacteria [32], these genes may produce the distinct tRNA modifications. Thus, some tRNA modification enzymes may have derived from other organisms and then evolved uniquely in the *T. acidophilum* genome. To clarify the evolution processes of tRNA modification enzymes in archaea, further studies will be required.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.09.021.

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